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Ultracentrifugation and Concentration of a Large Volume of Serum for HCV RNA During Treatment May Predict Sustained and Relapse Response in Chronic HCV Infection

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The ability to predict accurately a sustained response during therapy in patients with hepatitis C virus (HCV) infection is unavailable. The aim of this study was to differentiate, during therapy, patients who would relapse from those with a sustained response by ultracentrifugation for residual serum HCV RNA. Sixty-one specimens (from 32 patients) collected during interferon therapy were assessed by ultracentrifugation. All were negative using a quantitative polymerase chain reaction (PCR) (detection limit $\leq$ 100 copies/ml). One-milliliter aliquots were ultracentrifuged at 23,000 × *g* (160 min), and then the nucleic acid pellet was extracted, precipitated, and resuspended. Qualitative PCR was carried out in quadruplicate using two separate 5′UTR primer sets (8 results/specimen). A specimen was positive if $\geq$ 1 gels was positive compared to controls. At weeks 12 and 24, 9/9 (100%) sustained response patients were negative by ultracentrifugation. In the 23 relapse patients at week 12, 7/12 specimens were positive; at week 24, 7/14 were positive. Earlier time points could not differentiate the patients' eventual response to therapy. The predictive value of a positive ultracentrifugation test for relapse at week 12 or 24 was 100%. The predictive value of a negative test for sustained response was 62% and 50% at week 12 and 24, respectively. These preliminary results indicate that patients with an eventual sustained response will have no detectable serum HCV RNA by week 12 or week 24. A positive result is 100% predictive of relapse. ***J. Med. Virol. 57:351–355, 1999.***

KEY WORDS: hepatitis C virus; treatment; response; HCV RNA RT-PCR; ultracentrifugation

INTRODUCTION

The reported sustained biochemical and virological response rate observed with a standard 6- or 12-month course of interferon therapy is approximately 15–30% [Davis et al., 1989; Causse et al., 1991; Marcellin et al., 1991; Poynard et al., 1995, 1996; Hoofnagle and DiBisceglie, 1997]. Pretreatment variables reported to predict response to therapy in patients with chronic hepatitis C virus (HCV) infection are not consistent and the variables are predictive only in a population-based manner, providing little assistance for the management of individual patients [Davis et al., 1994; Martinot-Peignoux et al, 1995]. Published studies have shown that the presence of serum HCV RNA at early time points, particularly week 12 during treatment, accurately predicts nonresponse to therapy [Tong et al., 1997; McHutchison et al., 1998]. However, published studies suggest that the absence of serum HCV RNA early during therapy does not predict reliably, identify, and separate those individual patients who relapse after therapy from those with a sustained response [Kleter et al., 1993; Hino et al., 1995; Kohara et al., 1995; Orito et al., 1995; Hanley et al., 1996]. A test to separate these two groups would be advantageous for predicting the long-term outcome in sustained responders, and to potentially modify therapy in those likely to relapse.

In patients who are serum HCV RNA negative during therapy, we hypothesized that those who have an eventual sustained response should have no residual virus detected in serum by ultrasensitive techniques.

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In such patients, measurement of HCV RNA in a concentrated, large volume serum specimen during therapy when conventional polymerase chain reaction (PCR) is negative should theoretically also be negative. By comparison, those individuals who relapse virologically should, according to this hypothesis, have some residual viral RNA detectable in a large volume serum sample, even when serum HCV RNA is negative by PCR during therapy.

The aim of this study was to attempt to differentiate those patients with a sustained response from those with a relapse response. This differentiation was accomplished by carrying out an ultrasensitive PCR for residual serum HCV RNA during therapy in patients who were, by conventional PCR, negative for serum HCV RNA.

MATERIALS AND METHODS

Patient Selection

The group of 32 patients evaluated in this study had documented chronic HCV infection and had undergone a standard course of therapy with 3 million units three times weekly of interferon alpha 2b for 6 months. All patients had a sustained or relapse virologic response as defined below. The protocol was approved by the Human Subjects Ethics Committee and all patients gave informed consent. Chronic HCV infection was documented using standard criteria, including abnormal alanine aminotransferase (ALT) values for at least 6 months and biopsy-proven histological changes consistent with chronic hepatitis C. All patients tested repeatedly reactive for anti-HCV with the Ortho HCV second-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostics, Raritan, NJ), were positive with a recombinant-immunoblot assay (RIBA™, Chiron Corporation, Emeryville, CA), and had detectable serum HCV RNA by reverse transcription-PCR (RT-PCR). Other etiologic factors for liver disease were excluded in all subjects by serological testing, liver histology, and clinical history. All patients had compensated liver disease detected biochemically and clinically. The patients were followed throughout treatment and for a 6-month follow-up period. ALT values were assessed monthly throughout this time. Patients' serum was stored in 1.0-ml aliquots at −70°C within 2 hours of collection: at baseline, during treatment at weeks 1, 2, 4, and 12; at end of treatment week 24; and follow-up week 48. A total of 66 specimens from these 32 patients were thus available for testing by ultracentrifugation.

Biochemical response (BR) was defined using standard ALT criteria. A sustained response (SR) was defined when ALT values normalized during treatment and remained normal during follow-up; a response with relapse (RR) when ALT values normalized during therapy, but became abnormal during follow-up; and nonresponse (NR) when ALT values did not return to normal during treatment and follow-up. A virological response was defined as sustained when serum HCV RNA became undetectable during treatment by a multicycle PCR assay and continued through follow-up week 48; virological response with relapse when serum HCV RNA became undetectable during therapy but reappeared during follow-up.

Detection of Serum HCV RNA

HCV RNA was detected in serum samples in a blinded duplicate manner using a multicycle quantitative PCR assay as described previously [Tong et al., 1997]. The sensitivity of this assay is $\leq$ 100 copies/ml with an intrasample coefficient of variation of 26%. Ultracentrifugation of samples was then undertaken only if this assay was negative, and adequate serum was available.

Detection of Serum HCV RNA by Ultracentrifugation

Viral RNA was concentrated from the 1.0 ml patient serum aliquots by ultracentrifugation at 23,000 × *g* for 160 minutes at 16°C and extracted using a guanidium thiocyanate-phenol-chloroform mixture followed by ethanol-ammonium acetate precipitation [Chomczynski and Sacchi, 1987]. The precipitated RNA was centrifuged and the resulting pellet dried in a Centrivap console (Labconco, Kansas City, MO). The dry pellet was then resuspended in 30 μl of an Rnasin (Promega Corp., Madison, WI), dithiothreitol, and diethylpyrocarbonate-treated water mixture. The samples were kept at or below −20°C until RNA RT-PCR.

RT was carried out on the prepared RNA in eight separate reactions using random hexadeoxynucleotide primers (Pharmacia Biotech, Piscataway, NJ) (100 ng/μ) for cDNA synthesis. The mixture was heated to 70°C to denature RNA secondary structure and then cooled to room temperature to allow random primers to anneal to the RNA templates. Twenty microliters of a mixture containing M-MLV reverse transcriptase (USB, Cleveland, OH) and standard buffer components were then added and RT carried out at 40°C for 0 minutes. The cDNA was then heated to 90°C to inactivate reverse transcriptase, cooled to room temperature, and used immediately as template for the PCR. RT conditions were as follows: 3.3 U/μ reverse transcriptase, 5 mM $MgCl_2$, 50 mM KCl, 2.5 mM DTT, 1 mM dNTPs, 0.1% Triton X-100, 10 μg/ml acetylated bovine serum albumin (BSA), and 10 mM Tris-HCl, pH 9.0 for 1 hour at 40°C.

Seventy-five microliters of PCR mix was added to the entire RT reaction volume (26 μl) to a final $MgCl_2$ concentration of 1.65 mM in a total volume of 101 μ. A layer of mineral oil was added to prevent evaporation during thermocycling.

The PCR cycle consisted of annealing for 60 sec, extension for 60 sec, and denaturation for 60 sec, at 55°C, 74°C, and 94°C, respectively. After thermocycling, samples were submitted to a final 74°C final extension for 10 minutes. The thermocycling efficiency was assessed by satisfactory amplification of known copy number RNA standards included in each run. Two primer sets were used for the amplification, both from

the 5′ untranslated region of the HCV genome [Okamoto et al., 1990]. Both of these primer sets have been tested in our laboratory and have shown that they are highly conserved and will detect all known subtypes of HCV. Primer set 1: upstream 5′-GTG GTC TGC GGA ACC GGT GAG T-3′, downstream 5′-TGC ACG GTC TAC GAG ACC TC-3′ which produced a 196-bp product. Primer set 2: upstream 5′-CTG TGA GGA ACT WCT GTC TTC -3′, downstream 5′-CCC TAT CAG GCA CTA CCA CAA-3′, which produced a 256-bp product.

Both primer sets used in the ultrasensitive RT-PCR assay were shown to detect all of the known genotypes of HCV, as confirmed by comparisons to cloned HCV isolates from each of the different genotypes. The corresponding correlation coefficients (rho) for quantitation of known-quantity HCV clones from different genotypes including 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a were .9875, .9290, .9967, .9983, .9997, .9949, .9960, and .9988 respectively ($P < .001$ for all correlations) indicating a high level of quantitative efficiency for each HCV genotype.

The amplified cDNA was then electrophoresed in 3% agarose gel and detected by Southern blotting and immunostaining using a nonradioactive digoxingenin-labeled complementary DNA probe made in our laboratory. These procedures were carried out using automated instruments for PCR thermocycling, agarose gel electrophoresis, vacuum-transfer Southern blot, hybridization, and immunostaining. An internal control RNA was added to each sample at the first stage of the nucleic acid extraction. Amplification of this internal control ensures successful RNA extraction and confirms the sample does not contain PCR inhibitors. To minimize the possibility of post-PCR contamination, the pre- and post-PCR laboratories were located in separate buildings to ensure total segregation of personnel, equipment, and supplies. In each laboratory, ultraviolet (UV) lights were used to control contamination by depurination and/or cross-linking of any airborne or surface-bound PCR products. As a routine practice, all reagents used in the laboratory were prepared in single-use portions to prevent contamination of stock solutions. In addition, we included both reagent blanks and negative controls in each PCR run. The negative controls were of several types. All negatives were co-prepared with the samples and accompanied the samples through the entire PCR process. From prior experiments, the sensitivity of this assay is 13 copies/ml (95% CI of 8–18 copies/ml). This qualitative PCR was carried out in quadruplicate on four separate 3-μl aliquots. This yielded a total of eight individual results per specimen. A result was considered positive if one or more of the eight resulting gels was positive.

HCV Genotype Determination

HCV genotyping was carried out on biotin-labeled PCR products by hybridization to oligonucleotides directed against the variable region of the 5′UTR, and then immobilized as a parallel line on membrane strips (Line Probe Assay, InnoLiPa HCV II, Innogenetics, Brussels Belgium) [Stuyver et al., 1993]. Reactivity of the amplified fragments with one or more lines allowed recognition of six major genotypes and subtypes (i.e., 1a, 1b, 2a, 2b, 3a, 4, 5, and 6).

TABLE I. Baseline Characteristics in the 32 Patients Studied

Characteristic	Value
Sustained response	9
Relapse response	23
Age	43 ± 7 years
Sex	25/7 (M/F)
ALT (IU/L)	110 ± 55
HCV RNA (copies/ml)	$3.1 \times 10^6 \pm 3 \times 10^6$
Histology	
Chronic hepatitis	
Mild	10 (31%)
Moderate	11 (34%)
Severe	6 (19%)
Cirrhosis	5 (16%)
Genotype	3a(10),[a] 1a(7), 1b(7), 2(7), 4(1)

Results shown as means ± SD.
ALT, alanine aminotransferase; HCV, hepatitis C virus.
[a]Number in parenthesis is total number of patients.

Statistics

All statistical significance was assessed at the .05 level. Baseline data were descriptively summarized. Means and standard deviations were computed for all continuous data. Categorical data were summarized using frequencies. Assessment of positive and negative predictive value, specificity, sensitivity and likelihood ratios were completed as described previously [Friis and Stellers, 1996].

RESULTS

The mean age of the patients was 43 ± 7.5 years and 25 (78%) were males. As shown in Table I, all were typical HCV patients with moderately elevated ALT values and serum HCV RNA levels at baseline. Table I also shows their histological changes and genotype distribution. Nine of the 32 patients were sustained biochemical and virological responders, and the remaining 23 were relapse responders defined by both virological and biochemical criteria.

Figure 1 shows the results of the 25 specimens tested in the nine sustained response patients by week during therapy. Although the number of samples tested was small at early time points, the virus was usually present by the ultracentrifugation technique at weeks 1, 2, and 4. In comparison at week 12 and week 24 during therapy, all eight specimens tested by ultracentrifugation at each time point were negative at each of these time intervals. All nine patients were tested at week 12 or week 24 or both, and all results in these specimens were negative.

The results of the 36 specimens, tested by the ultracentrifugation technique in the 23 remaining relapse patients, is shown in Figure 2. Similar to the group of sustained responders, the responder relapsers had

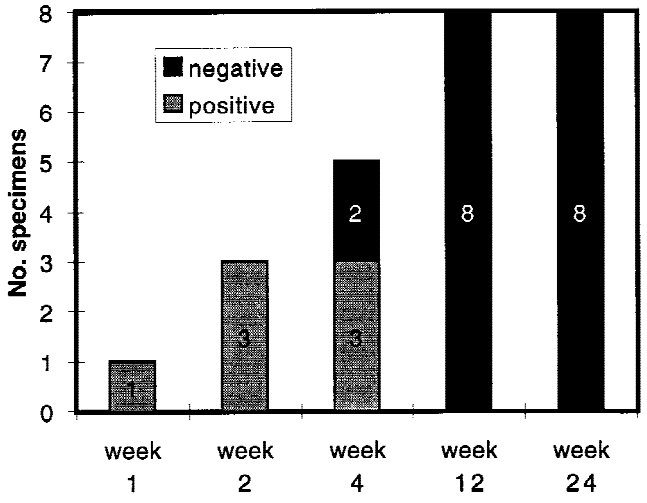

Fig. 1. Ultracentrifugation assay results in nine sustained response patients. Results are shown as positive and negative at each time point and the numbers on each bar indicate the number of specimens tested at each time interval with either a negative or positive ultracentrifugation result.

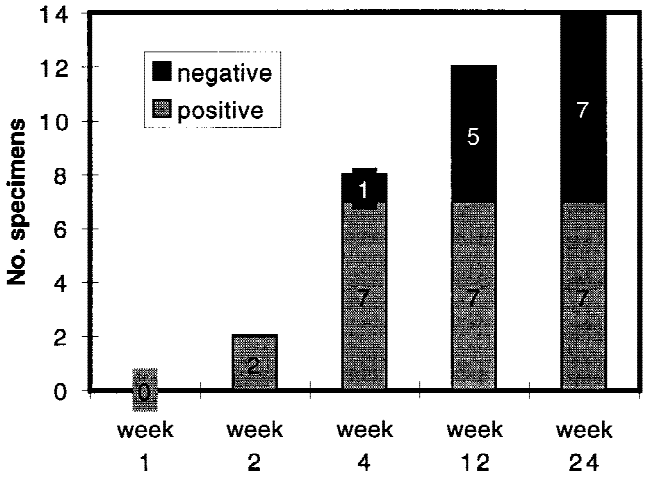

Fig. 2. Ultracentrifugation assay results in the 23 relapse response patients. Shown as a function of the week tested during therapy as either a negative or positive result (as shown).

early time points at weeks 1, 2, and 4 that revealed persistence of detectable virus by ultracentrifugation. However, by week 12, 7 of the 12 responder relapser specimens tested were positive and 5 were negative, and at week 24, 7 of the 14 responder relapser specimens tested were positive and 7 were negative as shown.

We then analyzed the virological response at week 48 as predicted by the week 12 ultracentrifugation results (Table II). Seven specimens were positive at week 12 by ultracentrifugation and all 7 patients were relapsers at week 48. Thirteen specimens were negative at week 12, 8 of whom were sustained responders at week 48 and 5 relapse responders. The predictive value of a positive ultracentrifugation test at week 12, or its ability to predict relapse, was thus 100% with a sensitivity of 58% and specificity of 100%. The predictive value of a negative test in predicting sustained response was 62%, and the overall accuracy was 75%. The likelihood ratio was 9.60 ($P = .002$). The evaluation of response at week 48 using week 12 ultracentrifugation data revealed that 7 patients were positive by ultracentrifugation at week 24, and all were relapsers at week 48. The predictive value of a positive test in predicting relapse was thus 100%, with 100% specificity but only 50% sensitivity. Fifteen samples were negative, 8 of which were eventual sustained responders at week 48. The predictive value of a negative test was thus 50%, with an overall accuracy of 65% (Table II). The likelihood ratio was 7.54 ($P = .006$).

TABLE II. Evaluation at Response Week 48 by Week 12 and Week 24 Ultracentrifugation Results

	Response at week 48	
Ultracentrifugation results	Sustained	Relapse
Week 12		
+	0	7
–	8	5
Week 24		
+	0	7
–	8	7

We set out subsequently to determine whether the negative ultracentrifugation results observed in relapse patients might be a function of the volume of serum tested. Two milliliters of sera from eight relapse patients and two sustained response patients during week 12 or week 24 of therapy were retested by ultracentrifugation. Both samples from sustained responders were also negative when this larger volume of serum was retested by this methodology. In the relapse patients, four of the eight results were negative but not interpretable, due to strong PCR inhibition within the samples as assessed by internal controls. Of the remaining four evaluable specimens, three (75%) did have detectable serum HCV RNA. The age of the serum specimen could not be correlated with negative ultracentrifugation results. In addition, we found no correlation between the results with the ultracentrifugation test and HCV genotype. Equal numbers of patients and tests were either negative or positive for each genotype.

DISCUSSION

This preliminary study assessing the ability of ultracentrifugation of a large volume of serum to detect residual HCV RNA, suggests that at week 12 and week 24 of therapy, we were able to detect residual serum HCV RNA in 50–60% of patients. These patients were negative during therapy by conventional serum PCR, but were eventual virological relapsers. The preliminary results suggest that the most appropriate time to perform such an assay is week 12 or 24 of therapy. Using this technique, all patients who were positive by ultracentrifugation at week 12 or 24 were classified as relapsers at 6 months follow-up, and all sustained responders were negative by ultracentrifugation at these

same time points. In addition, some relapse patients retested had detectable virus by ultracentrifugation when 2 ml, but not 1 ml, of serum was tested.

The potential to identify and separate relapse responders from sustained responders may have therapeutic implications. In these situations alternative therapies such as dose alteration or addition of other antiviral agents may be used in individual patients in the future, when proved effective in these clinical situations.

It has been suggested that patients with HCV who relapse after therapy have residual viral RNA too low to be detected by conventional assays. The results agree with this concept and indicate that residual serum HCV RNA can be detected in more than half of the relapse patients. However, some relapse patients were persistently negative when 1 ml of serum was tested by ultracentrifugation. There are a potential number of factors to explain these negative results in this patient population. Assay sensitivity and volume of serum tested could explain such negative results, and our preliminary findings with 2 ml of serum do indicate that the ability to detect residual virus is partially a function of assay sensitivity and/or volume of serum tested. Degradation of serum HCV RNA in stored specimens or varied assay sensitivity according to HCV genotype could also explain such negative results, but we found no correlation between the age of the specimens, HCV genotype, and these negative results. Finally, intracellular viral reservoirs (such as peripheral blood mononuclear cells) or actual degradation of RNA during ultracentrifugation are potential reasons for negative results that require further investigation.

These preliminary results require further expansion, verification, and prospective study in a larger cohort of patients with chronic HCV infection undergoing therapy. We do not advocate that this assay be used currently in a clinical setting, but the results obtained so far support our original hypothesis and require further careful study.

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